UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

THE INTERACTION BETWEEN MALARIA INFECTION AND PARENTAL AGE ON NEST DEFENCE BEHAVIOUR

CATARINA SOFIA GUIMARÃES DA CUNHA

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DOUTOR GEORGE THOMAS STILWELL

DOUTORA ILDA MARIA NETO GOMES ROSA

ORIENTADORA

DOUTORA ANNA QVARNSTRÖM

CO-ORIENTADOR

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To the rarest of all Joanas.

“Istes pa quamo!”

— Vítor Hugo

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ABSTRACT

Reproduction is a costly phase of the life cycle and in order to maximize reproductive success organisms are faced with trade-offs between different life history traits. While the presence of these trade-offs is obvious, there might be key differences in how they are solved within a population. Suppression or clearance of parasite infection requires investment in immunological responses, which leads to a reduction in investment in other costly functions. Parasite infection and parasite load may therefore cause changes in host's behaviour, including parental care behaviours, such as nest defence. However, whether infection affects these behaviours differently, depending on the age of the parent remains largely unexplored, especially in wild systems. Behavioural data and blood samples were collected from 206 collared flycatchers (*Ficedula albicollis*). We found a significant effect of the interaction between parental age and malaria infection on nest defence behaviours. One year old, infected birds had a lower frequency of nest defence behaviours than uninfected one year old ones, whereas the opposite pattern was present in older specimens. As previous studies suggest that young individuals may suffer more from acute infection, we argue that our findings imply that young infected individuals are in such a poor shape that they need to lower their activity level (i.e. we observe a direct cost of infection in term of lowered activity). In older individuals, a shift towards increased investment in current reproduction (i.e.terminal investment) is a likely explanation to increased risk-taking.

Keywords: Avian malaria; Collared flycatcher; *Ficedula albicollis*; nest-defence; terminal investment; Sweden; Uppsala

RESUMO

A fase reprodutiva tem um elevado custo energético no ciclo de vida e, de forma a garantir o sucesso reprodutivo, os indivíduos são confrontados com conflitos de escolha. Apesar da existência destes conflitos ser evidente, podem existir diferenças na forma como são resolvidos dentro de uma população. A supressão ou eliminação de uma infeção parasitária requer investimento em resposta imunitária, o que leva a menor investimento disponível para outras actividades de elevado custo energético.

A infeção e a carga parasitária podem, portanto, causar alterações no compor-tamento do hospedeiro, inclusive nos cuidados parentais, tais como a defesa do ninho. No entanto, permanece por explorar se a infecção parasitária afecta de forma diferenciada estes comportamentos dependendo da idade do progenitor, em particular em populações selvagens. Foram colhidos dados de observação de comportamento e colhidas amostras de sangue de 206 papa-moscas-de-colar (*Ficedula albicollis*). Os resultados revelam um efeito significativo da interação entre a idade dos progenitores e a infecção por malária nos comportamentos de defesa de ninho. Indivíduos juvenis infectados apresentam uma frequência de defesa de ninho inferior a indivíduos não infectados, enquanto o contrário se verifica em indivíduos mais velhos. Tal como estudos anteriores sugerem que indivíduos jovens podem ser mais afectados por infecção aguda, sugere-se aqui que estes resultados implicam que indivíduos jovens infectados se encontram em condição geral diminuída de tal forma, que necessitam diminuir o seu nível de atividade (i.e. observa-se um custo direto da infeção em termos de diminuição de atividade). Em indivíduos mais velhos, um aumento no investimento na reprodução (i.e. investimento terminal) pode explicar o aumento nos comportamentos de risco.

Palavras-chave: Malária aviária; papa-moscas-de-colar; *Ficedula albicollis*, defesa de ninho; investimento reprodutivo terminal; Suécia; Uppsala

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Summary of Activities

This work results from an internship that took place on the Swedish island of Öland. It is part of a bigger research project, belonging to the Qvarnstrom lab at Uppsala University, Sweden, stablished on the island since 2002. The project's biggest aim is to understand the mechanisms underlying speciation such as species barriers and formation. Hence it is stablished in a hybrid zone between 2 close related species, capable of hybridizing: the collared flycatcher (*Ficedula albicollis*) and the pied flycatcher (*Ficedula hypoleuca*). Several other smaller projects related to the main topic are developed in the field site, as was the case of this one. Data is collected during the breeding season, between May and July, since these are migratory species, that only come to Europe to reproduce. I followed 2 consecutive breeding seasons, on the years of 2018 and 2019, in a total of 6 months. The field season starts with the checking of the nest-boxes distributed throughout the field areas, a few days before the arrival of the animals. These are cleaned, repaired and replaced if needed. After, we follow the reproductive cycle, checking the boxes in specific times: we check the construction of nests, the laying of the first egg, the hatching of the eggs and the chicks with 6 and 12 days of age. We ring the birds, collect blood and general data such as measures of the tarsus, beak and wing span. Along this time, we observe the birds for behavioural studies, as it was the case of this project. By the end of the season we check the boxes to verify how many of the chicks fledged. I also preformed several dissections, necropsies and organ samples for other projects. After the field work was concluded, the samples were processed in the molecular lab. I extracted DNA from the blood samples and performed PCR with specific primers for malaria detection.

This work resulted in a poster presentation on the conference "TiBE 2018 | Host-Parasite Interaction" *(Appendix 2)*.

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List of abbreviations and symbols

AC → Alarm calls

AIC → Akaike information criterion

AvDP → Average distance to the predator

e.g. → *exempli gratia*

i.e. → *id est*

LPS → Lipopolysaccharides

MinDP → Minimum distance to the predator

PCR → Polymerase chain reaction

WF → Wing flicks

Part I: Avian malaria overview

Introduction

A diverse range of taxa are known to act as avian blood parasites, including filarids, namely microfilaridae (blood stages of filarial nematodes), flagellated trypanosomes and intracellular sporozoan parasites. This last group includes the Haemosporidians (Phylum Apicomplexa, Order Haemosporida), which are common blood parasites of reptiles, mammals and birds. Three closely related genera, *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are causal agents of avian malaria and are transmitted through the bite of different families of blood-feeding insect vectors (Order: Diptera) (Valkiunas, 2004). Haemosporidians are globally distributed and have been found in most bird species examined (Valkiunas, 2004). Diversity of haemosporidians is highest in tropical areas, but transmission commonly happens as far north as the Arctic Circle (Valkiūnas & Iezhova, 2018). Within each genus, diversity of species is high; for instance over 200 species of *Plasmodium* have been described (Martinsen & Perkins, 2013). Although the human malaria parasite belongs to this same genus, it belongs to a different phylogenetic group (Martinsen & Perkins, 2013; Outlaw & Ricklefs, 2011) and infections by avian-infecting *Plasmodium* species have not been reported in humans, thus avian malaria does not pose a zoonotic risk (Cox, 2010).

There are differences in prevalence, geographical distribution and host range of the parasites, which may be caused by host specificity due to parasite-host co-evolution, innate physiological differences that makes certain hosts more susceptible than others and availability of a vector population on host's habitat (Clark, Wells, Dimitrov, & Clegg, 2016; Lauron et al., 2015; Loiseau et al., 2012). Avian malaria infection is generally of little clinical significance, not being associated with events of mass mortality. However, in areas where previous exposure to the parasites was limited or nonexistent, it has caused significant devastation on bird populations (Derraik, Tompkins, Alley, Holder, & Atkinson, 2008). One of the best documented cases can be found in Hawaii. Between the 1800s and 1900s, about one-third of the 55 known species of Hawaiian honeycreepers became extinct due to the introduction of mosquitoes and invasive birds carrying *Plasmodium relictum*, together with other factors such as habitat loss and the introduction of nonnative predators (Atkinson & LaPointe, 2009). The remaining honeycreeper populations have since withdrawn to high elevation forests (above 1,500 meters), where malaria vectors survival is hindered by cooler temperatures (Liao, Atkinson, LaPointe, & Samuel, 2017; Samuel, Woodworth, Atkinson, Hart, & LaPointe, 2015). However, increase in temperature due to climate change and anthropocentric activities that leads to habitat disturbance, will likely allow parasites to expand their ranges, because it may increase vector habitat range. Consequently, instances of interactions between infectious and susceptible hosts also increases, thus influencing disease

dynamics (Atkinson & LaPointe, 2009). Therefore further understanding of the mechanisms of host-parasite interactions is particularly important.

Other well known cases involve captive animals, such as those in zoos, where there is combination of no previous exposure to the parasite and stress (related to captivity, handling, transport). The best documented cases involve cranes and penguins where sudden die-offs have occurred around the world (Botes, Thiart, Parsons, & Bellstedt, 2017). Malaria infection may be non clinically evident during outbreaks, and it is common to find dead birds without previous sign of disease. Sudden death may happen due to brain capillary blockage by the parasite (Ilgūnas et al., 2016). Clinical manifestations occur mainly in susceptible non-adapted avian species (as those mentioned) and are non-specific, occurring in several other diseases. These include loss of appetite, respiratory distress, lethargy, pale mucous membranes and anaemia where hematocrits may fall by more than 50% and regenerative haemolytic anaemia is observed (Gamble & Clancy, 2013). Neurological signs, such as motor incoordination, convulsions and paralysis, have been described in terminal states of severe forms of the disease (Grim et al., 2003; Valkiunas, 2004). Mortality rate can range from 50 to 90% in infected individuals, depending on species, parasite species and host age (Robinson, 2009). Necropsy findings include pulmonary oedema, hydropericardium and hepatomegaly, being possible to find the parasite on smears of spleen and liver (Robinson, 2009). Birds that survive the acute phase, become chronically infected which renders them immune to reinfection with the same species of parasite. Chronically infected birds perpetuate the life cycle by acting as reservoirs. In bird species that have been co-evolving with malaria parasites, virulence if often low and most individuals show no signs of infection (Valkiunas, 2004). Chronic infection produces generally mild symptoms of anaemia and relapses occur in stressful or demanding phases of the life cycle, such as low food availability, weakened immune systems, breeding and migration (Ricklefs et al., 2016).

Life cycle

The full understanding of the malaria cycle was only possible after 1897, when Ronald Ross described the developmental stages of the parasite in the vector, proving the transmission between infected birds and the mosquitoes, for which he was awarded a Nobel prize. Most of the knowledge accumulated on avian malaria cycle was gathered between 1930 and 1950, when these parasites were used as lab models to study human malaria. When rodent malaria parasites were found, they became the preferred lab model, due to their closer relationships to human *Plasmodium* species and their status as model lab organisms (Atkinson & Van Riper, 1991). The life cycle of avian haemosporidian parasites differs from those of mammals mainly due to the relatively low specificity host-parasite and vectors diversity,

which makes it a very interesting model to study host-parasite evolutionary theories (Atkinson & Van Riper, 1991). Some avian *Plasmodium* species have a much broader host specificity, being able of infecting hundreds of avian species in several different orders, using different vectors genera. However, *Haemoproteus* and *Leucocytozoon* tend to have a more restricted host range, being restricted to closely related species within the same host family (Atkinson & Van Riper, 1991).

Haemosporidians are obligatory heteroxenous, developing in two types of hosts: a vertebrate (intermediate) host and an invertebrate (definitive) host, where the sexual process of the cycle takes place. When female mosquitoes feed on the blood of an avian host, saliva injected into the host's tissues contains agametic stages (sporozoites) that proceed to invade reticuloendothelial cells (macrophages, monocytes and endothelial cells) and develop asexually into the first generation of exoerythrocytic meronts or schizonts (cryptozoites), which are most frequently detected in the lungs, liver, spleen, kidney and brain (Valkiunas, 2004). These meronts undergo asexual multiplication (merogony or schizogony) originating numerous smaller unicellular structures (merozoites). Merozoites induce new cycles of merogony greatly increasing the parasite population within the host. After rupture of the host cell, erythrocyte invasion follows. *Plasmodium spp*. and *Haemoproteus spp*. will form haemozoin granules (malarial pigment), a by-product from the incomplete digestion of the host cell haemoglobin. After invasion, *Plasmodium* species continues schizogony, forming erythrocyte meronts; *Haemoproteus* and *Leucocytozoon* species develop directly into gametocytes. These remain inside erythrocytes and halt their development until being ingested by a mosquito (Atkinson, Thomas, & Hunter, 2009; Valkiunas, 2004). There are two kinds of gametocytes: macrogametocytes or microgametocytes. After the invertebrate host feeds, each macrogametocyte originates one macrogamete, and each microgametocyte will undergo exflagellation and form eight microgametes. Fertilization occurs extracellularly and a mobile ookinete originates within 16–48 h, which proceeds to the epithelial layer of the midgut, where it becomes installed. Under the basal lamina, it rounds up and develops into an oocyst. During the oocyst development (sporogony), sporozoites are formed. After maturation these are released into the mosquito's haemocoele and then migrate to the salivary glands. Inoculation into the avian host occurs with saliva during the vector's blood meal (Atkinson et al., 2009; Valkiunas, 2004) (*see Figure 1*).

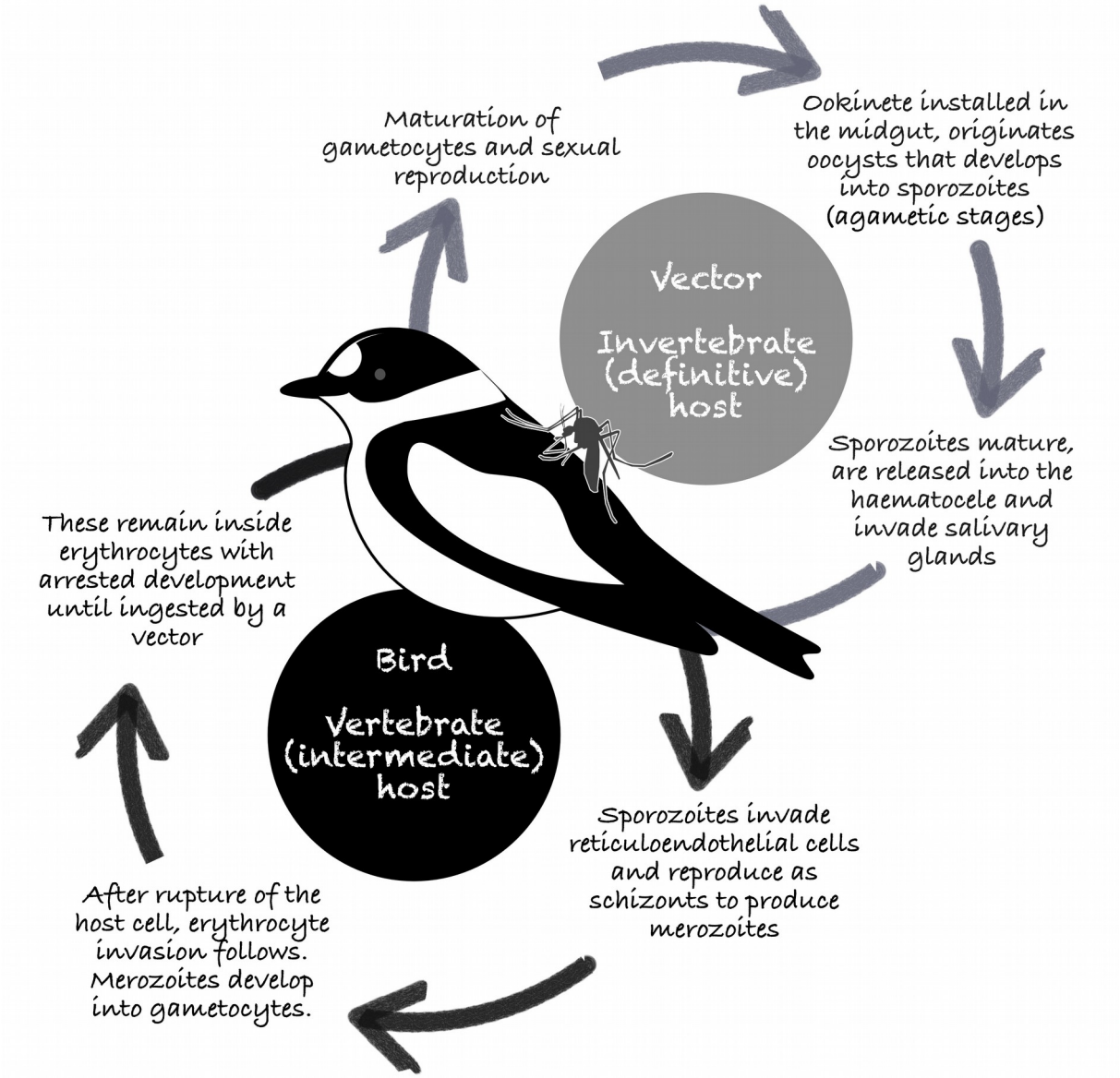

Figure 1. Schematic representation of a general life cycle of avian haemosporidian parasites (original).

Diagnosis

Haemosporidians can be detected based on blood-smear microscopy or molecular techniques. Both techniques encounter qualitative and quantitative limitations. Microscopic examination of Giemsa stained blood films is a cost-effective technique that provides an estimate of the parasitaemia intensity. Although some characteristics may help distinguish between the genera, a qualified and experienced professional is essential, since microscopic identification can be difficult and time consuming. Hence, estimates of diagnosis heavily rely

on quality of the slide preparation, the number of microscope fields analysed and observer expertise (Waldenström, Bensch, Hasselquist, & Östman, 2004). Furthermore, most avian haemosporidian surveys involve capture of wild birds that are generally at the chronic stage of infection, and during this stage parasites may remain undetectable in the blood stream. Microscopy reaches its limit of detection when parasitaemia falls below 40 infected red blood cells per microlitre of blood which equates to one parasite per 10,000 erythrocytes (Waldenström et al., 2004), which makes difficult to detect low intensity infections. Therefore, negative blood smears cannot be considered sufficient to rule out infection (Jarvi, Atkinson, & Schultz, 2002).

Molecular techniques such as polymerase chain reaction (PCR) assays have some advantages when compared to microscopy. For instance, they can be performed on hundreds of samples archived for years under varying storage conditions and are less constrained by technical expertise. These methods have significantly higher sensitivity than light microscopy, but may still fail to identify low-level parasitaemia (lower than 10^{-4}–10^{-5} parasites per erythrocyte) or co-infections (Braga, Silveira, Belo, & Valkiūnas, 2011; Krams et al., 2012; Valkiūnas et al., 2006), which are common and even predominate in many bird populations (Asghar et al., 2015; Atkinson et al., 2009).

Different protocols for PCR and nested PCR have been developed, targeting either 18S ribosomal subunit chromosomal gene (18S rRNA) or the cytochrome b mitochondrial gene (cyt-b) of the parasite. The detection rate of these protocols has been debated, with the mitochondria-targeted techniques being superior to those amplifying ribosomal DNA. These protocols developed by Waldenström et al., (2004) and Hellgren, Waldenström, & Bensch (2004), have gradually become the most widely used in the past decade. In short, these protocols consist in nested PCR that have a first PCR round that uses HaemNF and HaemNR2 primers and a second round that uses selective primers that amplify either 479 bp (HAEMF and HAEMR2) for *Plasmodium* and *Haemoproteus* or 480 bp (HAEMFL and HAEMR2L) for *Leucocytozoon* parasites. Although nested PCR is sensitive, it also has limitations. False negatives may occur due to insufficient concentration of parasite DNA or inadequate DNA extraction from the sample (Richard, Sehgal, Jones, & Smith, 2002). Moreover, PCR-positive results may result from DNA amplification of sporozoites or remnants of tissue meronts in cases where the parasite fails to develop a complete life cycle in the host (Levin et al., 2013; Valkiūnas et al., 2014).

Both molecular and blood smear analysis have advantages and disadvantages, for which considering them complementary may be of value (Braga et al., 2011; Valkiūnas et al., 2006).

Vectors

Haemosporidians are transmitted via a broad range of blood sucking dipteran insects hosts (Order: Diptera). Only females participate in the transmission cycle, since males are not haematophagus. The three genera infecting birds have different vectors. Parasites belonging to the genus *Plasmodium* are generally transmitted by mosquitoes belonging mainly to the three genera *Culex*, *Aedes* and *Culisetta*, unlike mammalian malaria parasites, which are transmitted mostly by *Anopheles* species (Atkinson & Van Riper, 1991). *Haemoproteus* species most often use *Culicoides* biting midges (Family:Ceratopogonidae) and louse flies (Family:Hippoboscidae) while *Leucocytozoon* is transmitted by black flies (Family:Simuliidae) (Atkinson & Van Riper, 1991; Valkiunas, 2004).

Part II: Parental care

Introduction

Parental care is part of some species' evolutionary strategy and it refers to a set of behavioural traits expressed by parents, that is directed towards their offspring and that enhances their offspring's fitness (Rosenblatt & Snowdon, 1996). Dependence of nestlings on parent care at hatching varies among species. While some young birds can leave the nest and begin finding their own food within hours of hatching, others are entirely dependent on their parents. Young birds can be generally categorized into two broad categories as either precocial, where nestlings leave the nest upon hatching and are able to feed themselves (such as megapodes); or altricial where nestlings hatch unfeathered and require feeding (such as passerines). There are species that fall in intermediate needs between these two extremes, such as is the case of penguins, gulls or owls, among others, that are semiprecocial (Ar & Yom-Tov, 1978; Temrin & Tullberg, 1995). Different modes of parental care have been recognised, among which the most common is bi-parental, especially in passerines, on which both male and female equally contribute to care for the offspring (≈81% of the species). In some cases, only the female (≈8%) or only the male (≈1%) is the caretaker, while some species adopt a cooperative breeding strategy, where care is provided not only from their parents, but also from additional group members (≈9%). In some cases (≈1%), species rely on others to raise their young (brood parasites) (Cockburn, 2006). The care provided involves feeding, brooding and protection from predators, which are of major relevance in altricial species, hatched without down and on which the main source of mortality among nestlings is predation (Clark & Wilson, 1981; Ricklefs, 1969). Because young birds from these species are unable to actively defend themselves, selection should act on parents to diminish predation pressures,

thus most species exhibit defence behaviours when potential predators approach a nest (Montgomerie & Weatherhead, 1988). These behaviours comprise calling, parental displays, approaching or even physical attacks on the predator (Gochfeld, 1984; Simmons, 1955). These serve the purpose of discouraging or distracting the predator, as is the case of alarm calls used as distractions, inclusively to lead predators away from the nest site (Gochfeld, 1984) and/or alert other birds in the vicinity, which might mob the predator (Curio, Ernst, & Vieth, 1978). Accounting for the frequency at which these happen allows to estimate intensity of nest defence (Andersson, Wiklund, & Rundgren, 1980; Blancher & Robertson, 1982; Greig-Smith, 1980).

Costs of nest defence and age-dependent trade-offs

While nest defence behaviours improve the fitness of the adult birds by increasing the chances of their offspring's survival, there are also some costs involved. Calling, displaying and approaching or even attacking the predator are not only time and energetically costly to the parent but may also infer the risk of getting hurt or killed by the predator (Montgomerie & Weatherhead, 1988). Parents are therefore faced with a trade-off between investment in defence of the current brood and investment in their own survival and therefore in future reproductive prospects.

Distinct life history strategies are adopted by different species, but for all of them, available resources in any particular environment are finite, which means that resources, such as time and energy, allocated to a certain trait diminish the amount available for another. This can be referred to as a trade-off – the allocation of resources in benefit of one trait, is detrimental to another. Resources' availability in the environment, together with individuals' ability to acquire and allocate them between traits, such as between reproduction and other costly activities, will determine the direction and magnitude of trade-offs. Consequently, in conditions of low energy availability, like low food abundance (Ebert & Hamilton, 1996; Ilmonen et al., 1999; Thomas et al., 2007) or during periods of high energy demand, such as investment in reproduction (Gustafsson, Nordling, Andersson, Sheldon, & Qvarnström, 1994; Thomas et al., 2007), the trade-off magnitude will be greater and more evident. When referring to reproductive investment, individuals can trade-off investment in current offspring (current reproduction), by the production of future offspring and the suppression of their own needs (future reproduction and current survival) (Richner & Tripet, 1999; Verboven & Tinbergen, 2002). As mentioned above, nest defence behaviour can also be studied in the context of life history theory and trade-offs between fitness components. While nest defence behaviour increases nest success (Andersson et al., 1980; Blancher & Robertson, 1982; Greig-Smith, 1980), decreasing the probability that a predator will cause damage to the offspring and increasing the offspring's chances of survival, it simultaneously comes with a cost to the parent, be-

cause it requires energy and it increases the probability of injury or death (Montgomerie & Weatherhead, 1988). Thus, parental investment in nest defence and important part of the overall parental time, energy and resources expended on offspring (Trivers, 1972), should be favoured by selection at the intensity that increases the survival chances of the offspring, but also that minimizes the chances of loss of future reproductive success due to injury or death of the parent. Therefore, the optimal time and energy allocated to nest defence depend on a variety of factors, such as the features of environment, the parents, the offspring, the predator or the nest (Trivers, 1972).

Parents' reproductive experience, gained along different breeding seasons, changes parents' perception for both costs and benefits of nest defence (Montgomerie & Weatherhead, 1988). A parent that is naïve to a particular predator is expected to be more cautious; hence intensity of defence behaviours should increase with increased experience, as the parent learns that it can increase nest defence without increasing the risk (Montgomerie & Weatherhead, 1988). On the other hand, experience also changes the perception of benefits. If by experience a parent recognises a predator as not posing a threat, nest defence will decrease, as it happens with repeated exposure to a model predator (e.g., Curio, 1975).

Several studies (East, 1980; Elliot, 1985; Redondo, 1989; Werschkul, 1979) report that intensity of nest defence varies according to how dangerous the predator is, with parents taking less risky defensive behaviours when confronted with more dangerous predators (Curio, 1975). Parents body condition also influences intensity of defence, as demonstrated in female Tawny Owls (*Strix aluco*) where individuals with poorer body condition defended their nests less intensively (Wallin, 1987). Sexual differences in nest defence intensity are expected, as confidence in parenthood or risk perception, among other factors, can be different for both parents (Montgomerie & Weatherhead, 1988; Redondo, 1989). Egg-laying requires a lot of energy from females, which means that after this period females might be weakened; thus a higher intensity of nest defence implies higher risks than for males during the same period (Reid & Montgomerie, 1985). Sergio & Bogliani (2001) found that, except during incubation, intensity of nest defence against a human intruder by female Eurasian Hobbies (*Falco subbuteo*) was higher than by males. However, higher investment by males seems to be the most common. In species with marked sexual body size difference, vulnerability to the same predator can be distinct. However, it should not be assumed that the smaller sex is always the most vulnerable. In the case of Snowy Owls (*Nyctea scandiaca*) and Rough-legged buzzard (*Buteo lagopus*), smaller body size seems to contribute to higher manoeuvrability, which facilitates males' nest defence behaviour (Andersson & Wiklund, 1987; Wiklund & Stigh, 1983).

The interaction between parents might also play a role in the intensity of nest defence. In biparental care species, males and females may take on separate nest-defence roles

allocating different amounts of energy in activities such as feeding or defending the nest from predators. Nonetheless, co-operation between the pair plays a role in the success of the nest, such as is the coordination of parents behaviour (Burtka & Grindstaff, 2015). Moreover, nest defence intensity may be higher for bi-parental care species if, in the case of the death of one of the parents, the other one is capable of taking care of the offspring by itself. On the contrary, in uni-parental care species, the caretaker incurs in greater costs, since its death means a lower chance of survival of the offspring, whereby investment in nest defence may be lower (Montgomerie & Weatherhead, 1988).

Nestling age has also been reported to influence intensity of parental defence (Montgomerie & Weatherhead, 1988; Sergio & Bogliani, 2001). Sergio & Bogliani (2001) reported a defence intensity increase from incubation to fledging, for both sexes, in Eurasian Hobbies (*Falco subbuteo*). This may be explained by the increase in reproductive value of the offspring to parents as nestlings age, due to an increase in expected fitness benefits for the adults. Higher quality broods and higher number of nestlings are also expected to increase adult fitness, whereby it is expected they would increase intensity of nest defence. Although some studies support these predictions (Knight & Temple, 1986; Sergio & Bogliani, 2001; Wallin, 1987), results are not always consistent (Curio & Regelmann, 1987; Regelmann & Curio, 1983).

Few studies have been done on the effect of parents' age on nest defence. However, it has been shown, that in long-lived species, such as gulls and geese, an increase in parental investment occurs (Montgomerie & Weatherhead, 1988). A decline in survival probability with senescence, explains the increase parental investment, since future reproductive chances are diminished (Pianka & Parker, 1975). However, in younger age classes, no change in parental effort is expected, since reproductive values do not considerably change between years. The same is predicted throughout the lives of most small, short-lived species, as is the case of passerines, where mortality is not age-dependent after the first year of life, as evidenced by Curio (1975) in Pied Flycatchers (*Ficedula hypoleuca*).

Infection status of the parents might also play a role on nest defence investment. There is evidence for the existence of a trade-off between investment in reproduction and immune function (Gustafsson et al., 1994; Knowles, Nakagawa, & Sheldon, 2009). Since both reproduction and immune response are energetically costly activities (Lochmiller & Deerenberg, 2000), individuals making a larger reproductive effort have less resources to invest in immune response, such as anti-parasite mechanisms. A decrease in immune response results in increased susceptibility to parasitism and greater parasitic infection levels, which can cause these individuals to have reduced survival and future reproduction prospects (Fisher & Blomberg, 2011). Haemosporidians are wide spread common agents of disease, with a wide range of hosts and making them good models to study host-parasite interactions. Among a

vast body of literature accumulated along the past century, there is evidence of malarial parasitic infection affecting a broad range of traits. Despite this, evidence for broadly negative effects of parasite infection is patchy. For instance, malaria-infected collared flycatchers have been shown to have no costs in timing of breeding and negligible costs of survival (Kulma, Low, Bensch, & Qvarnström, 2013). However, the price may be paid in reduced reproductive success, such as reduced clutch size as found by Ilmonen et al. (1999), in house martin (*Delichon urbica*). On the other hand, Szöllősi et al. (2009) found no effect of malaria infection on the growth rate and fledging size of collared flycatcher nestlings.

Although much of the effect of infection on host behaviour traits is still unexplored, a few studies point to an effect of infection on host's behaviour. Risk-taking behaviour has been studied by Marinov et al. (2015), who found evidence that higher risk-taking nightingales (*Luscinia megarhynchos*) were more likely to present protozoan parasites in blood than less risk-taking individuals. Garcia-Longoria et al. (2015) found that malaria infected house sparrows (*Passer domesticus*) had more intense escape behaviours. Effect of infection on locomotor activity (number of jumps) has also been tested by (Mukhin et al., 2016), in wild passerines. Experimentally malaria infected siskins (*Spinus spinus*) showed a decrease in locomotor activity with higher parasitaemia (i.e. infection intensity). Regarding nest defence, a study on Tengmalm's owls (*Aegolius funereus*) observed negative association between prevalence of blood Trypanosoma parasites and nest defence (Hakkarainen, Ilmonen, Koivunen, & Korpimäki, 1998). However, the influence of malaria on nest defence behaviour attending on how parents' age might influence the resolution of this resource allocation has yet to be studied.

Methods

Ethical statement

Ethical permission for this work was provided by the Swedish Board of Agriculture (Linköping Animal Ethics Board DNR 21-11).

Study species — *Ficedula albicollis*

The collared flycatcher (*Ficedula albicollis*) is a small, migratory passerine songbird (Order: Passeriformes, Family:Muscicapidae) (*see Figures A1 & A2*). It breeds across deciduous woodlands in central and eastern Europe, from north-eastern France to south-western Russia and with isolated populations in central Italy and on the Swedish Baltic islands of Gotland and Öland which have been recently colonised (Qvarnström, Rice, & Ellegren,

2010). The species migrates to southern East Africa (Gwinner, 1990), just south of the equator, to overwinter, flying through Italy and the Balkans, over the Mediterranean Sea and the Sahara Desert. It feeds on invertebrate prey caught either in flight or gleaned from vegetation. The breeding season starts in early May and lasts until the end of June. Collared flycatchers are cavity nesters and will readily use nest-boxes when provided (Alatalo, Carlson, & Lundberg, 1988). Females lay between 5 to 7 eggs, incubating them for 12 to 14 days and chicks are ready to fledge after 15 days (Schluter & Gustafsson, 1993). As a species, its conservation status if of Least Concern (BirdLife International, 2019).

The study was conducted on the swedish population of Öland, which has been monitored since 2002. The long-term monitorization allows access to data such as pedigrees and individuals' age, which is scarcely available in wild systems. Besides, collared flycatchers are an important study system for avian malaria due to a vast quantity of knowledge gathered along the years, being used as model species.

Figure A1. Male collared flycatcher. Picture by Carolina Segami

Figure A2. Female collared flycatcher with eggs. Picture by Carolina Segami

General field procedures

Data was collected during breeding seasons (May–June) of 2013, 2014 and 2016. Approximately 2500 nest-boxes are distributed through 25 areas, that comprise oak woodlands, coniferous (pine) and mixed forests. A total of 206 individuals (108 females and 98 males were captured in the nest with the help of a trap placed in the boxes, ringed (for

identification purposes), weighed, measured (wing, beak and tarsus length) and aged according to plumage features as described by Svensson (2010) as being 1-year old or ≥2-years old. A blood sample was collected from the brachial vein with a 27G sterile syringe needle, into capillary tube and placed into a 95% ethanol filled eppendorf tube or smeared on a FTA® card, Whatman™.

Females were captured during the incubation period and males during the nestling feeding period. Nest-boxes were periodically monitored to determine reproductive parameters such as lay date (first day of egg-laying), clutch size and hatch date (first day of egg-hatching). On day 6 after hatching, nestlings were ringed and weighed and a blood sample was collected. On day 12, nestlings were re-weight and tarsus was measured. By the end of the season, nest-boxes were inspected one last time to determine number of successfully fledged nestlings.

Behavioural data

It has been demonstrated in pied flycatchers that similar nest defence intensity is shown when presented to taxidermy or live predators (Curio, 1975). Thus, a taxidermy model of a Eurasian sparrowhawk (*Accipiter nisus*) was placed on the top of each nest-box and an observer stood in a hideout approximately 20 meters from the focal nest, for a period of ten minutes. Every minute, we registered for each parent, if alarm calls (AC) and wing flicks (WF) occurred or not, *at least once*, with measuring scores varying from 1 to 10 (1 = alarm/flick at least once in one of the 10 minutes; 10 = alarmed/flicked at least once in every minute). In addition, the distance to the predator was measured during each minute from which minimum distance (MinDP) was registered and average distance calculated (AvDP).

Observations took place when nestlings were 8 to 10 days old, and only nests in which both parents were observed were taken into the data analysis (*see Figure 2*).

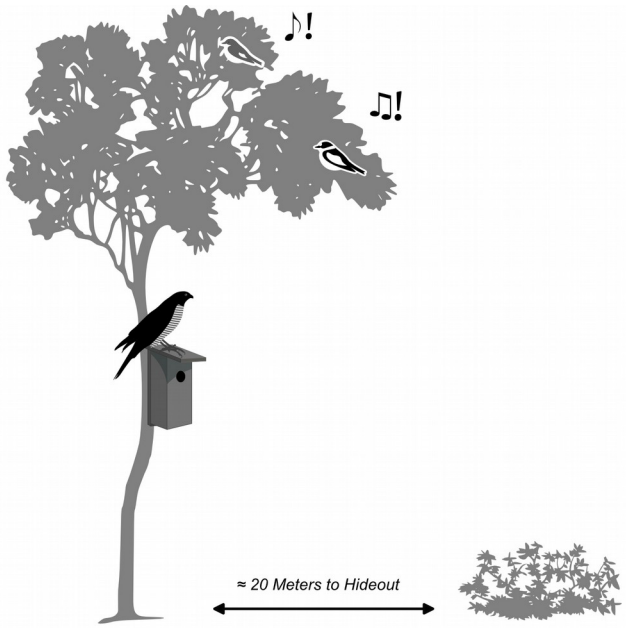

Figure 2. Schematic representation of field set-up for behavioural data collection.

Avian malaria diagnosis

Blood stored in FTA® Cards was extracted with the method described by (Smith & Burgoyne, 2004) for avian blood and blood stored in ethanol eppendorfs was extracted using a high salt extraction protocol (Aljanabi & Martinez, 1997).

Extracted DNA was used as a template for PCR assay. We used a nested PCR protocol described by Hellgren et al., (2004), which targets a segment of the parasite's cytochrome b gene (Plasmodium and Haemoproteus). To reduce the likelihood of false negatives and positives, negative controls (ddH_20) and positive controls were run during the PCR stages. PCR products were scored as present (infected) or absent (not infected) after running 1.5% agarose gels stained with GelGreen. To prevent cross-contamination, separate areas of the lab were used for DNA extraction, pre- and post-PCR work.

Variation across the breeding season

Nest defence behaviours have been shown to vary across the breeding season (Biermann & Robertson, 1981; Curio, Regelmann, & Zimmermann, 1984; Regelmann & Curio, 1983). We therefore considered the possible effects of this external factor alongside with the two factors related to the condition and quality of the parental bird (i.e. age and malaria infection status), which were our main focus of interest.

Data analysis

Data was statistically analysed with R version 3.5.1 (R Development Core Team, 2018). To analyse behavioural data, we first tested for correlation between the different behaviours measured, using Pearson's correlation test.

To build the statistical model for continuous data (AvDP and MinDP), we used linear models (R function lm). For counted data (AC and WF) whose distribution was other than normal, we used generalized linear models (R function glm), assuming poisson distribution. As we were interested in any relation between malaria and age, we included in the model an interaction between these two factors.

We used the Akaike information criterion (AIC) to select the model that better fitted the data, successively eliminating fixed effects or nonsignificant interactions. On a first approach to our data, we used generalized linear mixed models (R package lme4) (Bates et al, 2015). As random effects we defined area, observer and year, as we thought these might have a non-systematic, unpredictable influence on the data. However, none of these random effects explain any variance on the data, whereby the use of linear models was more adequate. The fixed effects that resulted in a best fitted model were sex, age, malaria and day of the breeding season. The 1st day of the breeding season corresponds to the 1st of May of each

year. However, individuals adjust their breeding season to yearly differences, such as differences in temperature or humidity. Therefore, the variable day of the season was corrected to the mean of hatching day of each year.

All the plots, including prevalence of malaria infection among sex, year and age, were generated with the R package ggplot2 (Wickham, 2016).

Results

General pattern of malaria infection prevalence

The prevalence of malaria infection varied between the three study years, with a significant low infection prevalence in 2016 when compared both with 2013 ($z_{1,205}$=-4.913, p<0.001) and 2014 ($z_{1,205}$=-2.003, p=0.045) (*see Table 1, Figure 3A*). Although not significant, a slightly higher number of one-year old birds were infected than older birds (*Figure 3B*). No difference between sexes was found (*Figure 3C*).

*Table 1. Generalized linear model output, testing for the effect of year on malaria prevalence. Significant effects are in bold, *P-value <0.05, ***P-value <0.001.*

Variable	Estimate	Std.Error	Z	Pr(>|z|)
(Intercept 2013)	0.47	0.3291	1.428	0.153
Year 2014	-0.9808	0.5349	-1.834	0.067.
Year 2016	-1.9278	0.3924	-4.913	**<0.001*** **
(Intercept 2014)	-0.5108	0.4216	-1.212	0.226
Year 2016	**-0.9469**	**0.4727**	**-2.003**	**0.045***

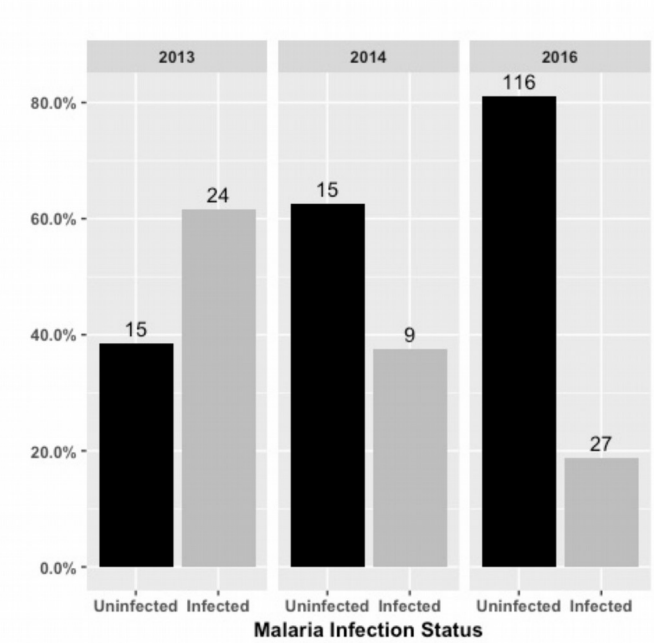

Figure 3A. Malaria infection prevalence throughout the three study years Sample size for each group indicated on each bar.

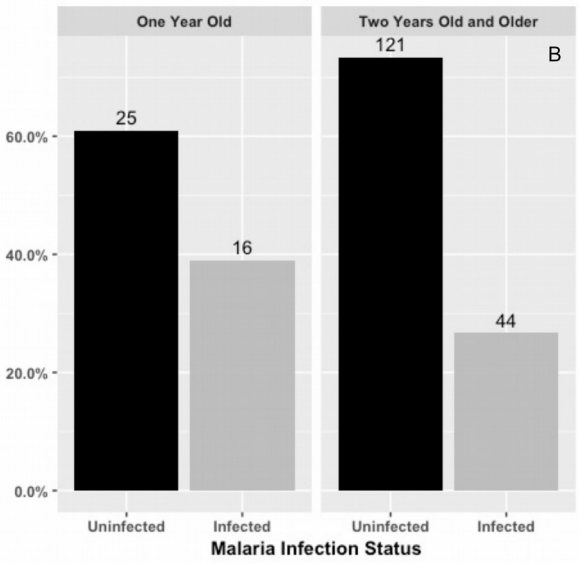

Figure 3B. Comparison of malaria infection prevalence between age groups. Sample size for each group indicated on each bar.

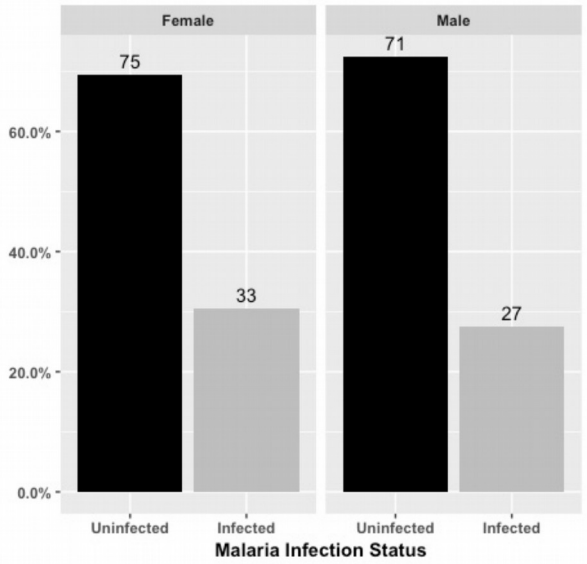

Figure 3C. Comparison of malaria infection prevalence between sexes.Sample size for each group indicated on each bar.

Behavioural data

Correlation between measured behaviours

A strong positively correlation was found between AC and WF (*Pearson's correlation, r2 = 0,74, p= 2.2*$^{-16}$) and MinDP and AvDP (*Pearson's correlation r2 = 0,82, p*= 2,2^{-16}).

Alarm calls & wing flicks behaviours

As expected, due to the fact that the two variables are strongly correlated, very similar variance is observed between AC and WF. No significant effect of malaria infection status on number of alarm calls was found, but a trend for a lower number of wing flicks when birds are infected is present (*p*=0.054, *see Table 3, Figure 5A*). Old birds alarm more (*p*=0.037) and flick their wings more (*p*=0.012) when infected, and younger birds alarm and flick less, as shown by the significant effect of interaction between age and malaria *(see Tables 2 & 3, Figures 4C & 5C)*. However, in general, younger birds significantly alarm more than older birds (*p=0.034, see Table 2, Figure 4A*). There was no effect of sex in number of alarm calls, however there is a trend for males to flick their wings more than females (*p*=0.063). Both behaviours significantly increased along the breeding season (*p*<0.001, *see Tables 2 & 3, and Figures 4B & 5B*).

*Table 2. Generalized linear model output, testing the effect of age, sex, malaria infection status and day of the breeding season on alarm call behaviour. Model selection was based on Akaike's Information Criterion (AIC).Variable Day of the breeding season corrected for mean of hatching day of year. Significant effects are in bold, *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

Variable	Estimate	Std.Error	Z	Pr(>|z|)
(Intercept)	**1.879606**	**0.08124**	**23.136**	**<0.001*** **
Malaria Infection Status				0.1237
Infected	-0.195024	0.126687	-1.539	
Age				**0.034***
≥ 2 Year Olds	-0.18078	0.086792	-2.083	
Sex				0.7469
Male	0.018563	0.057523	0.323	
Day of the breeding season	**0.034164**	**0.008589**	**3.978**	**<0.001**
Interaction (Malaria x Age)				**0.037***
Infected * ≥ 2 Year Olds	0.300907	0.144129	2.088	

*Table 3. Generalized linear model output, testing the effect of age, sex, malaria infection status and day of the breeding season on wing flick behaviour. Model selection was based on Akaike's Information Criterion (AIC).Variable Day of the breeding season corrected for mean of hatching day of year. Significant effects are in bold, *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

Variable	Estimate	Std.Error	Z	Pr(>|z|)
(Intercept)	**1.60252**	**0.091366**	**17.539**	**<2e-16*** **
Malaria Infection Status				0.054.
Infected	-0.279594	0.14496	-1.929	
Age				0.131
≥ 2 Year Olds	-0.145884	0.096709	-1.508	
Sex				0.063.
Male	0.117956	0.063387	1.861	
Day of the breeding season	**0.039664**	**0.009471**	**4.188**	**<0.001**
Interaction (Malaria x Age)				**0.012***
Infected * ≥ 2 Year Olds	0.409373	0.163192	2.509	

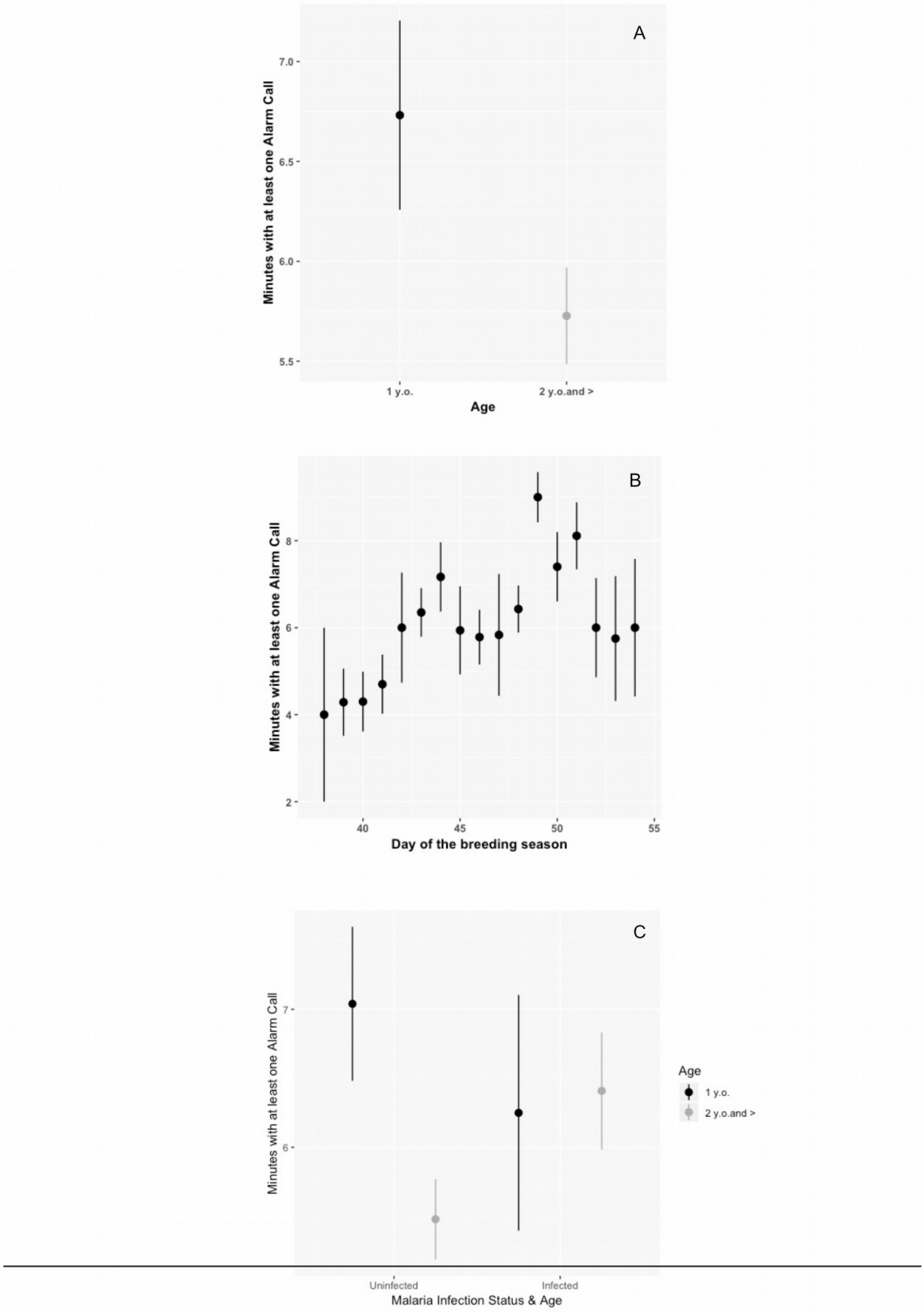

Figure 4. Comparison of number of minutes with at least one alarm call (mean ± SE) between different age groups (A). Figure 4B represents the number of minutes with at least one alarm call (mean ± SE) along the days of the breeding season. Figure 4C represents a comparison between different malaria infection status individuals, taking into consideration the age group (malaria infection status and age interaction).

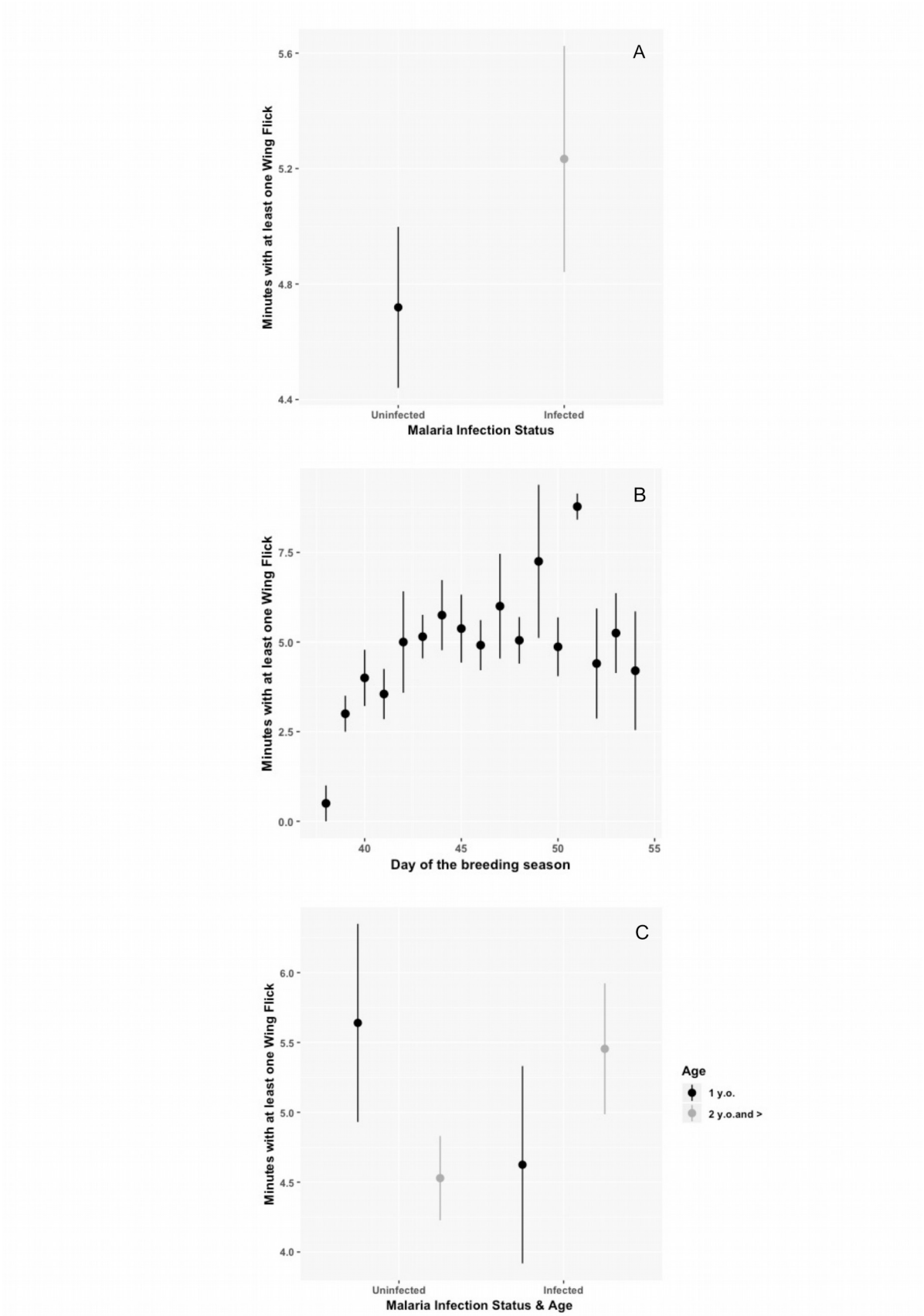

Figure 5. Comparison of number of minutes with at least one wing flick (mean ± SE) between different malaria infection status individuals (A). Figure 5B represents the number of minutes with at least one wing flick (mean ± SE) along the days of the breeding season. Figure 5C represents a comparison between different malaria infection status individuals, taking into consideration the age group (malaria infection status and age interaction).

Average & minimum distances to predator

Malaria infection status and age had no effect on the distances birds would get to the predator. However we found a significative difference between males and females, with males getting significantly closer than females (p=0.005, *see Table 4, Figure 6A*). As expected due to high correlation between the two variables, the same happens for average distance (p=0.031, *see Table 5, Figure 6B*). The average distance to the predator significantly decreased along the breeding season (p=0.003, *Figure 6C*).

*Table 4. Generalized linear model output, testing the effect of age, sex and malaria infection status on minimum distance to the predator. Model selection was based on Akaike's Information Criterion (AIC).Variable Day of the breeding season corrected for mean of hatching day of year. Significant effects are in bold, *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

Variable	Estimate	Std.Error	Z	Pr(>|z|)
(Intercept)	**2.06965**	**0.43656**	**4.741**	**<0.001**
Malaria Infection Status				0.502
Infected	0.45113	0.67051	0.673	
Age				0.26
≥ 2 Year Olds	0.52474	0.46478	1.129	
Sex				**0.005****
Male	-0.82772	0.29013	-2.853	
Day of the breeding season	0.05997	0.043	1.394	0.165

*Table 5. Generalized linear model output, testing the effect of age, sex and malaria infection status on average distance to the predator. Model selection was based on Akaike's Information Criterion (AIC). Variable Day of the breeding season corrected for mean of hatching day of year. Significant effects are in bold, *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

Variable	Estimate	Std.Error	Z	Pr(>|z|)
(Intercept)	**3.80329**	**0.49753**	**7.644**	**<0.001**
Malaria Infection Status				0.455
Infected	0.5721	0.76415	0.749	
Age				0.982
≥ 2 Year Olds	0.01212	0.52968	0.023	
Sex				**0.031***
Male	-0.72042	0.33065	-2.179	
Day of the breeding season	**0.14712**	**0.04901**	**3.002**	**0.003****

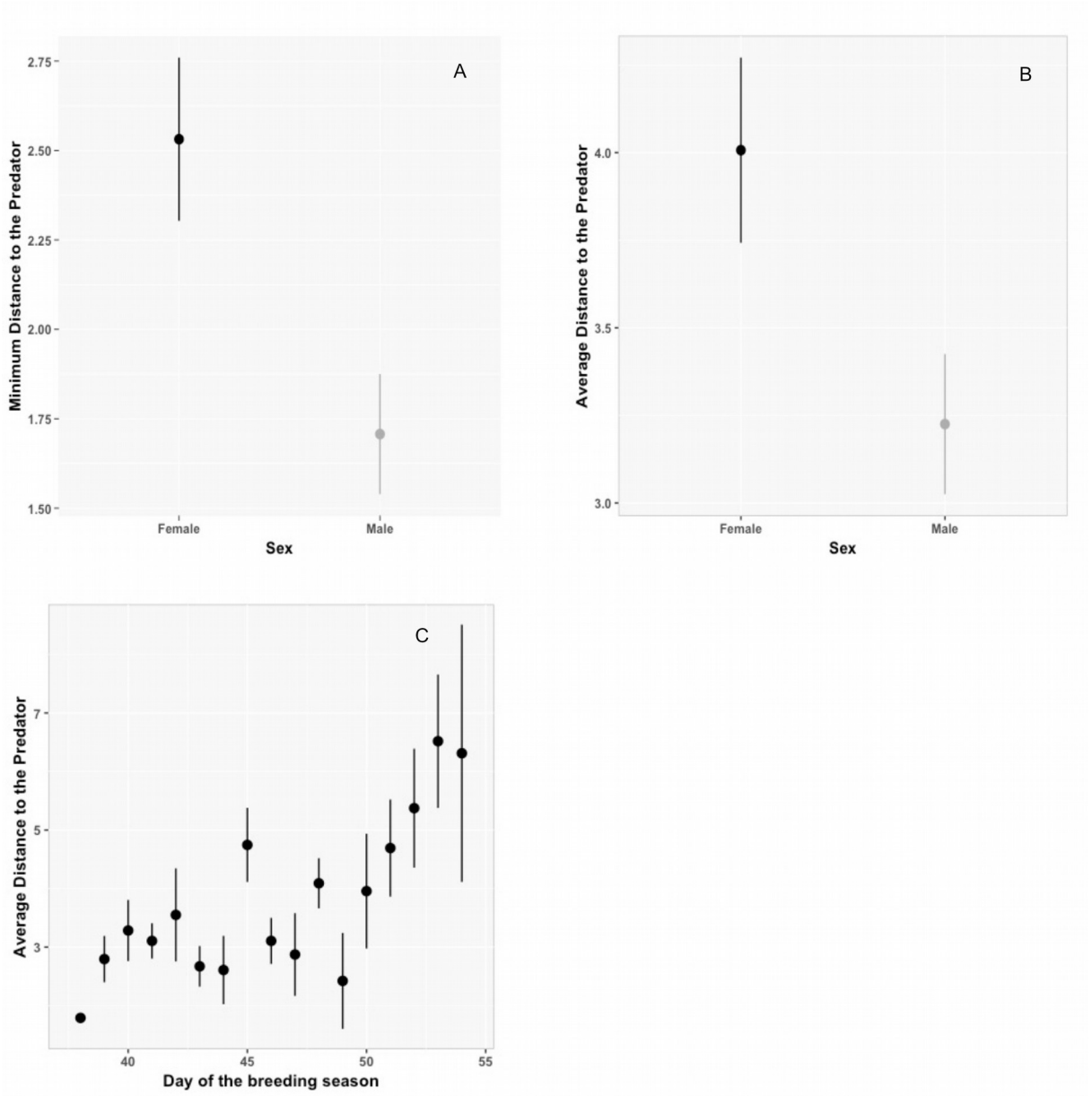

Figure 6. Comparison of the minimum (6A) and average (6B) distances to the predator (mean ± SE) between males and females. Figure 6C represents the distance (meters) to the predator (mean ± SE) along the days of the breeding season.

Discussion

We found that male collared flycatchers were getting significantly closer to the predator dummy that we had placed at their nest-box as compared to females. This finding supports previous studies on anti-predator behaviour in other avian systems, where males have been observed to defend nests at a higher intensity than females during the nestling stage, when presented with a predator (Gibson & Moehrenschlager, 2008; Michl, Török, Garamszegi, & Tóth, 2000). As previously mentioned, a series of factors may explain differential intensity in nest defence behaviour between the sexes. Risk perception may change for females right after egg-laying, since they are overcoming a considerable drain of energy, resulting in them taking fewer risks than males (Montgomerie & Weatherhead, 1988; Redondo, 1989). Female collared flycatchers are occasionally seen raising nestlings by themselves, unlike males (personal communication, Anna Qvarnström). This supports the prediction that the sex unable to raise young alone should invest more in nest defence. It has also been debated how plumage colour may influence nest defence behaviour. Baker & Parker (1979), predict that brightly coloured birds will most often be found to suffer less from predation than comparable more cryptic birds. If that is to be true, being the more conspicuous sex may also contribute to higher defence intensity in male collared flycatchers. Moreover, sex hormones are known to play a role in aggressiveness, which can also play a part in a more intense defence from males, whom have higher levels of testosterone (Wingfield, Ball, Dufty, Hegner, & Ramenofsky, 1987). Although it may seem that males might be more dedicated to defending the nest, the case might be that both sexes invest similar amounts of energy, with different strategies, and female strategy may include avoid revealing the location of the nest (Zimmermann & Curio, 1988).

We found no evidence for differences in how closely the predator was approached between the age groups, but the number of minutes with alarm calls differed. Montgomerie & Weatherhead (1988), predicted that with increased experience of the parent the intensity of defence would also increase, since individuals learn that they can engage more intensively in defence without increasing the risk. The results seem to be in contrary to this prediction, since young, and hence less experienced birds alarmed significantly more than older birds. However, young birds also tended to keep further away from the predator. We propose that older, more experienced birds, have learned that engaging in more risky behaviour, such as approaching closer to the predator, may be more efficient than alarming further away from the nest. Thus, they, in fact, engage in more intense defence, as predicted by Montgomerie & Weatherhead (1988), whereas younger birds, unfamiliar with the predator, stay further away, alarming. An alternative proposed by the same authors is that learning may have the

opposite effect, decreasing intensity of defence. Hence, older birds might have learnt that alarming is a poorly efficient strategy towards that predator, thus decreasing the behaviour.

It has been previously demonstrated in collared flycatchers that risk taking behaviours are not influenced by parasite infection (Garamszegi et al., 2015). Our results are partly in accordance to this finding, since we found no effect of malaria infection on distance to predator for either males or females. However, we found significant interactions between age and malaria infection on the other measured nest-defence behaviours. First year, infected birds had a lower frequency of defence behaviours than uninfected first year birds, whereas the opposite pattern was present in older birds. As previously mentioned, organisms must distribute their resources among competing fitness components in a trade-off fashion. Thus, when facing an immune challenge, the higher energy demand to sustain the immunologic reaction, can lead to a decrease in investment in other costly activities, as reproduction. On the other hand, if the presence of infection internally cues individuals to reduced prospects of survival and future reproduction, this can lead to an increase in reproductive investment. This is called the 'terminal investment hypothesis', and is a prediction from life-history theory, which suggests that if the chances of surviving to reproduce in the future are low, animals should invest more in current reproductive output, as that breeding bout will likely correspond to the last opportunity to produce offspring for a given individual (Clutton-Brock, 1984). Interestingly, our results suggest patterns of terminal investment in the defence behaviours of collared flycatchers. Young, infected birds likely spend less time defending their offspring as they can afford to trade-off current reproduction for future reproduction. Older, infected birds are engaging in higher intensity defence behaviour as they are investing more into current reproduction. Examples of this phenomenon are documented in several species: Derting & Virk (2005), showed that male white-footed mice (*Peromyscus leucopus*) treated with sheep red blood cells display larger testes than control animals. A positive relationship between malaria infection status and reproductive performance was found by (Podmokła et al., 2014) in blue tits (*Cyanistes caeruleus*). In collared flycatchers terminal investment has been described previously (Mariusz Cichoń, Sendecka, & Gustafsson, 2003; Part, Gustafsson, & Moreno, 1992). By measuring feeding bouts and daily energy expenditures, Part et al., (1992), showed that old female collared flycatchers invested more in brood care than younger females. The cost of this investment was paid in reduction of their survival probability resulting from an increased allocation of resources to current reproduction.

The biased investment away from survival and towards immediate reproduction is likely to depend on the relative life span expectancy (Williams, 1966), but the mechanisms behind which cues trigger a modification of their resource allocation pattern remain unclear. Intrinsic factors, such as age, and also extrinsic ones, such as predation, food shortage, and parasitism potentially influence mortality rate (Bonneaud, Mazuc, Chastel, Westerdahl, & Sorci,

2004). Some of these factors have been shown to alter reproductive investment, as is the case of predator pressure, which can temporarily decrease parental investment as prey species invest more into future reproduction (Korpimaki, Norrdahl, & Valkama, 1994). Immune system activation has also been proposed as a cue factor for a rapidly diminishing life span (Grenfell, Dobson, & Moffatt, 1995), thus being a cause of terminal investment. Hamsters treated with lipopolysaccharides (LPS) to elicit an immune response and induce symptoms of declining survival prospects, had an increase in testes size and seminal vesicles, more testicular sperm, and higher testosterone concentrations at the end of the experiment (Weil, Martin, Workman, & Nelson, 2006); also when challenged with LPS, reproductive success of mature blue-footed booby (*Sula nebouxii*) males fell, while that of old males showed a strong increase (Velando, Drummond, & Torres, 2006). Using inert antigens, a natural population of house sparrows female treated with Newcastle disease virus vaccine laid larger replacement clutches than those treated with a vehicle when initial clutches were first removed (Bonneaud et al., 2004). Although how the kind or dose of antigen can influence these patterns is still obscure (Bonneaud et al. 2004), these studies of immune challenges on reproductive output point to it being sufficient as a cue to produce an alteration in patterns of reproductive investment. Taking this into consideration, our results allow us to conclude that malaria infection is causing a pattern of terminal investment in our population, which can be revealed by alteration in nest defence behaviours.

Several authors have focused on the study of variation of behaviour occurring along the breeding season (Biermann & Robertson, 1981; Curio et al., 1984; Regelmann & Curio, 1983). Most observations of parental care behaviours point to an increase in defence response during the breeding cycle, which also seems to be the case in our population. The reason for this phenomenon has been considerably debated, but no clear conclusion has been achieved (see Montgomerie & Weatherhead, 1988; Redondo, 1989 for detailed discussion). Other than finding the same pattern, our data also does not allow us to take any conclusions on why the increase in nest defence occurs. Seasonal breeding allows for optimization of survival of the offspring, which, in the case of temperate zone species, are born and raised during the fraction of the year when energy resources are relatively abundant (Prendergast, 2005). Although resources may be plentiful, reproduction is still a high energy demanding activity. Hence, nesting should be concentrated around peaks of food abundance, because as season progresses, nestlings survival probabilities may be lower due to decrease in food supply (Carlisle, 1982). Thus, birds breeding late in the season should have a lower residual reproductive value than birds nesting earlier. This could be reflected in an increase in defence for early broods, and a decrease for broods late in the season, which have lower reproductive value. Although not markedly, some authors have reported results that support this argument (Biermann & Robertson, 1981; Curio et al., 1984;

Regelmann & Curio, 1983). Redondo (1989), also argues that if predation risk increases along the season, variation in defence levels may be reflecting predation risks and not parents' reproductive value. We did not have enough predation data to test this argument, as such it was not possible to explain our results taking predation into consideration. Although for the experimental data each nest was visited only once, other field activities occur along the season that imply several visits to each nest.

Since our experiment was performed in a natural population of birds, there was some general patterns of variation in occurrence of malaria infection. We found a considerably lower prevalence in 2016, which was a year with low levels of rain and humidity, but also it follows 2014 and 2015, also dry years (*Appendix 1*). The majority of the strains infecting the population are European (Jones et al., 2018), suggesting that infection of this population occurs mainly in the study area in Europe and not during overwintering in Africa. Since vectors breed in stagnant water collections, the right amount of rainfall, that allows pooling is often important. Relative humidity affects malaria transmission through its effect on the activity and survival of vectors being better under high humidity conditions. An average monthly relative humidity below 60% decreases mosquitoes survival in such a way that malaria transmission considerably decreases (Yamana & Eltahir, 2013).The differences in infection found between the years, although not significant, might also be explained by different stages of infection. Since infection tends not to be eliminated but instead becomes chronic, some of the older birds might be infected but with parasitemia levels inferior to detection level.

Some studies have approached differences in malaria prevalence between sexes. In great tits (Podmokła et al., 2014), males were found to have higher prevalence of infection when taking care of increased number broods. The authors cannot disentangle if this is due to higher exposure to the vectors, since males fed their offspring more often than females, or higher susceptibility to the parasite due to higher energy demands, but gives proof of differences in prevalence according to sex. In our population of collared flycatchers, we also found a trend for differences in prevalence between sexes, with higher prevalence of infection in females (unpublished data). While males acquire the initial territory and help provision offspring, females invest heavily in egg production and are wholly responsible for incubation of eggs and newly-hatched nestlings (M. Cichoń, 2000). Both increased time exposed to the vector while incubating eggs and brooding hatchlings, and also increased energy demands for egg-laying, might render them more susceptible to infection.

Summary

In general, our results match the predictions and reveal the existence of an interaction between malaria infection and parental age on nest defence behaviours. Young infected individuals engage in lower intensities of nest defence behaviours, while old infected individuals engage in higher. We argue that young individuals reduce their activity due to reallocation of limited energetical resources towards their immune system. Meanwhile, old birds shift their investment towards current reproduction in a form of terminal investment. This study brings attention to the role that parasites play in parental investment and to the importance of taking into consideration different age classes in wild populations.

Further steps

In further studies and to shed a bit more of light on how infection interferes with defence behaviour, it would be relevant to quantify malaria infection, allowing the understanding of possible differences in nest defence behaviour between individuals with different infection levels. Moreover, several predator species, other than the Eurasian sparrowhawk are present on Öland and are known to be important predators for hole-nesting birds, including pine martens (*Martes martes)*, least weasels (*Mustela nivalis)* and great spotted woodpeckers (*Dendrocopos major)* (Dunn, 1977; Sonerud, 1985; Wesołowski, 2002). Further studies on nest defence behaviour using different predators would also be interesting to see whether flycatchers are able to adapt their response. The ability to do this has been demonstrated in other species such as black-billed magpies (*Pica hudsonia*) *a*nd tits *Paridae* (Buitron, 1983; Tvardíková & Fuchs, 2012).

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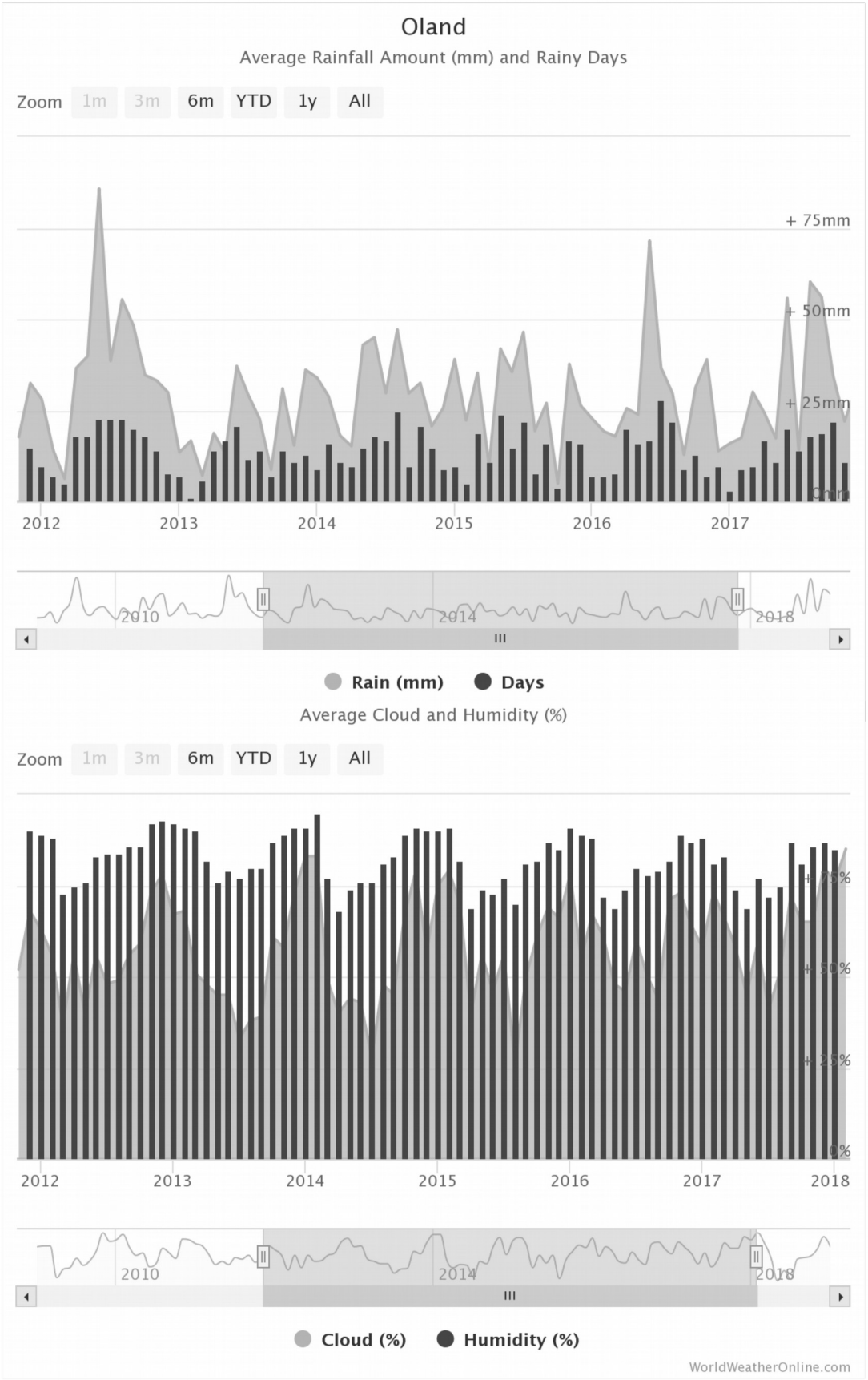
Appendixes



Appendix 1. Average rainfall amount (mm) & rainy days (A) cloud and humidity on Öland, between 2012 and 2018.

UPPSALA UNIVERSITET

Old Birds Increase their Nest Defence Behaviour when Infected with Malaria

CATARINA CUNHA*, WILLIAM JONES, ANNA QVARNSTRÖM
CAT.CATARINACUNHA@GMAIL.COM

FRAMEWORK

Reproduction and suppression or clearance of **parasite infection** are costly activities for hosts.

To maximize reproductive success organisms are faced with **trade-offs** between different life history traits.

Key **differences** may exist in how these trade-offs are solved **within a population.**

There is evidence that **parasitic organisms interfere** with their hosts in several ways, including causing changes in hosts' **parental care behaviour**, such as nest defence.

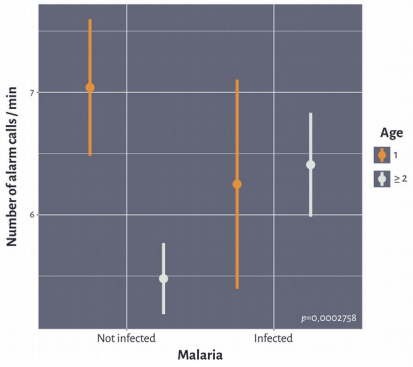
It remains unexplored whether infection affects these behaviors differently depending on the **age of the parent**, especially in wild systems.

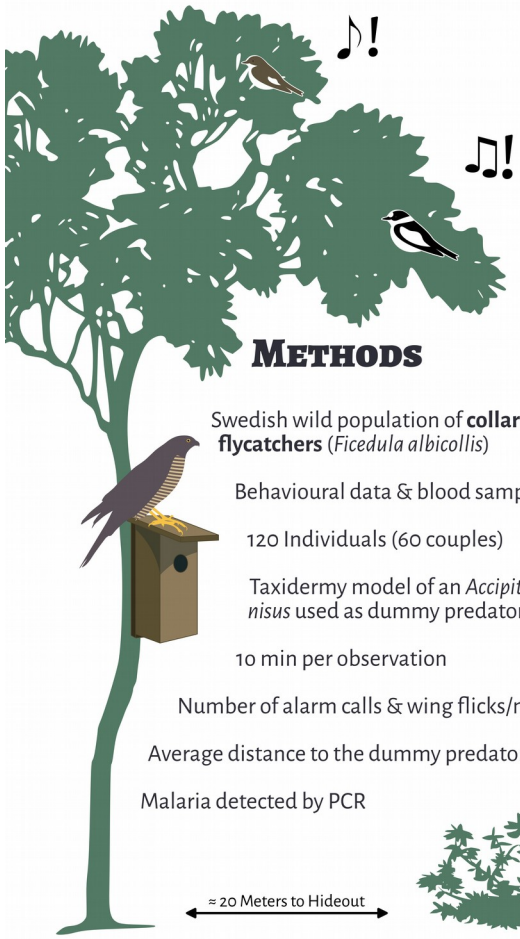
RESULTS

Older infected birds show a significantly **higher number** of **alarm calls** and **wing flicks**.

Average distance to the dummy predator shows no statistical significance.

(however, younger birds tend to keep a higher average distance than older birds)





METHODS

Swedish wild population of **collared flycatchers** (*Ficedula albicollis*)

Behavioural data & blood samples

120 Individuals (60 couples)

Taxidermy model of an *Accipiter nisus* used as dummy predator

10 min per observation

Number of alarm calls & wing flicks/min

Average distance to the dummy predator

Malaria detected by PCR

≈ 20 Meters to Hideout

INTENSITY OF NEST DEFENCE
when infected

YOUNGER BIRDS HAVE LOWER DEFENCE

Lower number of alarm calls & wing flicks

Tend to keep greater distance to the predator

Higher chances of future survival

Investment in future reproduction

OLDER BIRDS HAVE HIGHER DEFENCE

Higher number of alarm calls & wing flicks

Tend to get closer to the predator

Lower chances of future survival

Investment in current reproduction

***Trade-off** between current reproductive success and survival*

Appendix 2. Poster presented on TiBE 2018 | Host-Parasite Interactions